Structural basis for the efficient phosphorylation of AZT-MP (3′-azido-3′-deoxythymidine monophosphate) and dGMP by Plasmodium falciparum type I thymidylate kinase

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Plasmodium falciparum is the causative agent of malaria, a disease where new drug targets are required due to increasing resistance to current anti-malarials. TMPK (thymidylate kinase) is a good candidate as it is essential for the synthesis of dTTP, a critical precursor of DNA and has been much studied due to its role in prodrug activation and as a drug target. Type I TMPKs, such as the human enzyme, phosphorylate the substrate AZT (3′-azido-3′-deoxythymidine) inefficiently compared with type II TMPKs (e.g. Escherichia coli TMPK). In the present paper we report that eukaryotic P.fTMPK (P. falciparum TMPK) presents sequence features of a type I enzyme yet the kinetic parameters for AZT-MP phosphorylation are similar to those of the highly efficient E. coli enzyme. Structural information shows that this is explained by a different juxtaposition of the P-loop and the azide of AZT-MP. Subsequent formation of the transition state requires no further movement of the P/TMPK P-loop, with no steric conflicts for the azide moiety, allowing efficient phosphate transfer. Likewise, we present results that confirm the ability of the enzyme to uniquely accept dGMP as a substrate and shed light on the basis for its wider substrate specificity. Information resulting from two ternary complexes (dTMP–ADP and AZT-MP–ADP) and a binary complex with the transition state analogue AP₅dT [P-(5′-adenosyl)-P₅-(5′-thymidylic) pentaphosphate] all reveal significant differences with the human enzyme, notably in the lid region and in the P-loop which may be exploited in the rational design of Plasmodium-specific TMPK inhibitors with therapeutic potential.

Key words: drug target, ligand complex, malaria, Plasmodium falciparum, thymidylate kinase (TMPK).

INTRODUCTION

An essential step in the biosynthesis of the nucleotide dTTP is the phosphorylation of dTMP to dTDP. This reaction is catalysed by TMPK (thymidylate kinase; EC 2.7.4.9) in a magnesium-dependent manner with ATP as the preferred phosphate donor. Cellular production of dTTP is a highly regulated process [1,2], which is co-ordinated with DNA replication in the cell cycle, and TMPK has a central role in the control of the dTTP pool size in eukaryotic cells. The enzyme has been widely studied partly due to its participation in the pathway that leads to the activation of a number of prodrugs, including AZT (3′-azido-3′-deoxythymidine) and acyclovir for the treatment of AIDS and herpes simplex virus infections respectively [3,4]. TMPK is also being investigated as a drug target in its own right and specific inhibitors of the orthologous enzymes from Bacillus anthracis and Mycobacterium tuberculosis have been developed [5,6].

TMPK is found in all organisms requiring de novo pyrimidine synthesis, and crystal structures of the enzyme from a number of different species have been determined [7–11]. The protein is a homodimer with a subunit fold consisting of a five-stranded parallel β-sheet surrounded by 7–11 α-helices. The nucleotide substrates are bound in a groove on the surface of the subunit, which is characterized by a number of conserved sequence motifs containing structural elements required for substrate recognition and catalysis, namely the so-called ‘P-loop’ [12] G(X₅)GKS/T, the critical loop Asp-Arg-Xaa (DRX) motif (Figure 1a) and a ‘lid’ that partially encloses the phosphate donor and is implicated in catalysis [8]. Crystal structures of TMPKs with different combinations of ligands have revealed several conformational states of the protein: an open state in the absence of ligand (where the lid is disordered); a partially closed state in the presence of dTMP (where the lid is disordered in most structures, but ordered in the Mycobacterium tuberculosis enzyme); and a fully closed state in the presence of both co-substrates (where the lid is ordered). Co-crystallization with bi-substrate analogues, such as AP₅dT [P-(5′-adenosyl)-P₅-(5′-thymidylic) pentaphosphate], which mimic the ATP–dTMP or ADP–dTDP states [13] has given additional insight into the reaction co-ordinate [14,15].

After extensive functional and structural studies on TMPK from several species, a general consensus with regard to the mechanism has been achieved. There is a good overview of the mechanism based primarily on structural and functional studies of the human enzyme [15]. Owing to the complexity of the reaction, there are small differences between species, such as the movement of the P-loop and the lid during catalysis so as to re-orient specific...
Figure 1  Sequence and structure of TMPK proteins

(a) Structure-based amino acid sequence alignment of selected TMPK proteins (Pf, P. falciparum; Hu, human; Sc, Saccharomyces cerevisiae; Mt, Mycobacterium tuberculosis; Ec, E. coli). Strictly conserved residues are coloured red and well-conserved residues are coloured yellow. The locations of the P-loop, critical loop and lid region in the P. falciparum enzyme are highlighted by red triangles, red stars and a blue bar respectively. The secondary structure elements of PfTMPK are shown above the sequence alignment. This Figure was generated using ESPript [42]. (b) Stereo view of protomer A of PfTMPK–TMP–ADP (ribbon) showing the dTMP substrate and ADP product as spheres, the P-loop (red), lid region (green), the critical loop (magenta) and the two sodium ions (I and II, grey spheres). The Figures were made with CCP4mg [43].

basic side chains to stabilize the transition state complex. These may well have impacts on the specificity and relative kinetics of the enzyme. Sequence variation, particularly in the lid region, is associated with structural alterations around the nucleotide-binding site and relocation of catalytic side chains, leading to speculation that the subtelities of the mechanism vary between species [8]. Comparison with related systems has given some general insights into the mechanism. In a similar fashion to adenylate kinase, which has the same overall topology as TMPK, the two nucleotide substrates are believed to bind to the enzyme via a random Bi Bi mechanism [16]. It is agreed that phosphate transfer occurs via an associative, rather than a dissociative, mechanism, such that the migrating phosphate is bound to both substrates in the transition state [17]. The accumulating negative charge on this phosphate in the transition state is proposed to be stabilized by arginine side chains and one or more magnesium ions.

The TMPKs have been classified into two groups [8]. Type I enzymes, which include the human, yeast and Plasmodium proteins, have a basic residue in addition to the lysine residue in the P-loop [Arg\(^\text{18}\) in PfTMPK (P. falciparum TMPK)]. Type II enzymes, including that from Escherichia coli, lack this basic residue in the P-loop; a basic residue from the lid appears to exert a similar function. TMPKs from different species have varying AZT-MP (AZT monophosphate) phosphorylation efficiencies and comparative structural and biochemical studies have shown the structural basis for proficient phosphorylation. TMPKs from E. coli and various viruses are able to phosphorylate AZT-MP very much more efficiently than the human enzyme [18]. The reason for this appears to be the displacement of a catalytically important arginine side chain on the P-loop of the human enzyme in response to AZT-MP binding. In contrast the E. coli enzyme, where the P-loop arginine residue is absent, can accommodate AZT-MP without seriously affecting its catalytic properties [8]. The focus of the present study was to explore the potential of PfTMPK as an anti-malarial drug target. We present a kinetic analysis of PfTMPK in combination with the crystal structure of four ligand complexes: dTMP–ADP, AZT-MP–ADP, AP5dT and dGMP–ADP. The latter structure provides a structural rationalization for the broader specificity of the Plasmodium enzyme. Two recent studies by Kitade and colleagues present results on the expression and kinetics [19] and on the ligand binding, modelling and mutagenesis [20] of PfTMPK, and together with new kinetic data they interpreted in terms of the crystal structures presented in the present paper. The present results confirm that the enzyme has structural and catalytic properties distinct from other members.

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EXPERIMENTAL

Materials

AZT-MP, 3′-dGMP and AP, dT were purchased from Jena Biosciences. Other nucleotides, including ATP (magnesium salt), were purchased from Sigma–Aldrich. Oligonucleotides were synthesized at the Analytical Services of the López-Neyra Institute of Parasitology and Biomedicine. *P. falciparum* strain 3D7 was provided by J. M. Bautista of the University Complutense of Madrid, Madrid, Spain. Human erythrocytes and frozen serum were obtained from Centro Regional de Transfusión Sanguínea-SAS (Granada, Spain).

Parasite culture

*P. falciparum* strain 3D7 was cultured by the method of Trager and Jensen [21] with minor modifications. Cultures were maintained in fresh group-O-positive human erythrocytes suspended at 5% (v/v) haematocrit in RPMI 1640 (Gibco BRL) containing 10% (v/v) human serum, and 3 g of glucose, 45 (v/v) haematocrit in RPMI 1640 (Gibco BRL) containing 10% (v/v) human serum, and 3 g of glucose, 45% (v/v) haematocrit in RPMI 1640 (Gibco BRL) containing 10% (v/v) human serum, and 3 g of glucose, 45% (v/v) haematocrit in RPMI 1640 (Gibco BRL) containing 10% (v/v) human serum, and 3 g of glucose, 45% (v/v) haematocrit in RPMI 1640 (Gibco BRL) containing 10% (v/v) human serum, and 3 g of glucose, 45% (v/v) haematocrit in RPMI 1640 (Gibco BRL). 10 μl of binding buffer [50 mM Tris/HCl, pH 7.5, 50 mM KCl, 5 mM MgCl2, 0.2 mM NADH, 1 mM DTT, 1 mM phosphoenolpyruvate, 4 units of pyruvate kinase, 4 units of lactate dehydrogenase, 50 μM dTMP, 1 mM ATP and 0.85 μg of recombinant enzyme. When \( K_i \) and \( V_{\text{max}} \) values for the different nucleotides were determined, a range of concentrations were tested using the standard assay. TMPK activity (1 unit) was defined as that which produces 1 nmol of NADH per min per mg of enzyme. \( K_i \) values were obtained from the expression \( IC_{50} = K_i (1 + [S]/K_{\text{m}}) \) that, for competitive inhibitors, relates the concentration of inhibitor which inhibits activity by 50% (IC50) with the \( K_i \) value. \( K_m \) is the Michaelis–Menten constant for dTMP and \([S]\) is the concentration of dTMP used in the reaction (50 μM).

Expression of *PTMPK* in *E. coli*

DNA encoding full-length *PTMPK* (633 bp) was amplified by RT (reverse transcription)–PCR from total RNA obtained by standard procedures from parasites released from infected erythrocytes by saponin lysis [22]. The primer TKEcoRI (5′-GGAA TTCTTA TGACCACAAAA TTAA-3′) was used to synthesize the corresponding *tmkp* cDNA, in the RT reaction, and TKNdeI (5′-GCATATGCTAGTGATAAAAAAAA-3′) and TKEcoRI were used in a second PCR step to amplify the full coding sequence with NdeI and EcoRI restriction sites placed at the 5′- and 3′-ends respectively for cloning into the *P. falciparum* cloning vector pET28a (Novagen) so as to append a His6-affinity purification tag and a thrombin cleavage site to the N-terminus of the *PTMPK* peptide sequence. The resulting pET-PTMPK plasmid was used to transform the *E. coli* strain BL21 (DE3) and *PTMPK* expression was induced by adding 1 mM isopropyl \( \beta \)-D-thiogalactoside (Roche) and incubating for 4 h at 37°C or by means of auto-induction [23] for a period of 20 h. The cultures were collected by centrifugation (6600 g for 10 min) and frozen at −80°C until use.

Purification of recombinant *PTMPK*

For purification of *PTMPK*, cell pellets were resuspended in 10 ml of binding buffer [50 mM Tris/HCl, pH 7.5, containing 300 mM NaCl, 10 mMimidazole, 1 mM DTT (dithiothreitol) andprotease inhibitor cocktail (Pefabloc SC; Roche)] to a give final concentration of 2 mM. Pefabloc SC-protector solution (Roche) was also added to prevent non-specific covalent attachment of the protease inhibitor to the protein (a problem encountered during initial purification trials). Cells were disrupted by sonication on ice (six 30 s bursts at 50% maximum amplitude using a MSE Soniprep 150 sonicator) and centrifuged at high-speed (14000 g for 30 min at 4°C). The cleared lysate was applied to a HiTrap™ Chelating HP Column (GE Healthcare) equilibrated with binding buffer. The column was washed with binding buffer containing 20 mM imidazole, and was developed with a linear gradient of 20–500 mM imidazole. Fractions containing the enzyme were pooled, passed through a PD-10 column (GE Healthcare) and eluted in 50 mM Tris/HCl, pH 7.5, containing 20 mM NaCl, 1 mM DTT and 50% (ν/ν) glycerol. For structural work, the protein was further purified by gel filtration on a HiLoad 16/60 Superdex 75 prep-grade gel-filtration column (GE Healthcare) pre-equilibrated with 50 mM Tris/HCl, pH 8.5, and 200 mM NaCl. The protein concentration was determined using the Bradford method [24], with BSA as a protein standard. The purity and molecular mass of the purified protein were established by PAGE and electrospray MS. The protein was concentrated to 40 mg·ml⁻¹ and stored at −80°C.

Activity assays

Recombinant *PTMPK* enzyme activity was assayed using a spectrophotometric method by coupling the formation of ADP to the oxidation of NADH in reactions catalysed by pyruvate kinase and lactate dehydrogenase [25]. Assays for inhibition determination were performed in a final volume of 1 ml and a standard assay contained 50 mM Tris/HCl, pH 7.4, 50 mM KCl, 5 mM MgCl2, 0.2 mM NADH, 1 mM DTT, 1 mM phosphoenolpyruvate, 4 units of pyruvate kinase, 4 units of lactate dehydrogenase, 50 μM dTMP, 1 mM ATP and 0.85 μg of recombinant enzyme. When \( K_i \) and \( V_{\text{max}} \) values for the different nucleotides were determined, a range of concentrations were tested using the standard assay. TMPK activity (1 unit) was defined as that which produces 1 nmol of NADH per min per mg of enzyme. \( K_i \) values were obtained from the expression \( IC_{50} = K_i (1 + [S]/K_{\text{m}}) \) that, for competitive inhibitors, relates the concentration of inhibitor which inhibits activity by 50% (IC50) with the \( K_i \) value. \( K_m \) is the Michaelis–Menten constant for dTMP and \([S]\) is the concentration of dTMP used in the reaction (50 μM).

Crystallization, data collection and data processing

To remove the His6-tag from the protein prior to crystallization, biotinylated thrombin (Novagen) was added to a solution containing 1 mg·ml⁻¹ *PTMPK* in 50 mM Tris/HCl, pH 8.5, 200 mM NaCl and 25 mM CaCl2 (1 unit of thrombin per μg of protein). This solution was gently swirled at room temperature (22°C) for 24 h. The thrombin was then removed from the reaction mix by immobilization on streptavidin–agarose according to the manufacturer’s instructions, and a final nickel-affinity chromatography step was used to remove any uncleaved *PTMPK*. Electrospray MS analysis confirmed the identity and purity of the product and the presence of a Gly-Ser-His extension at the N-terminus of the protein (24.973 kDa). Needle-shaped crystals (up to 50×50×400 μm in size) of four different nucleotide complexes of *PTMPK* were obtained using hanging-drop vapour diffusion, with a 1:1 mixture of protein solution and reservoir solution in the drop (see Supplementary Table S1 available at http://www.BiochemJ.org/bj/428/bj4280499add.htm). Prior to data collection, the crystals were vitrified at 120 K in an appropriate cryoprotectant solution (Supplementary Table S1). X-ray data sets for the four protein complexes (see Table 3) collected at the ESRF (European Synchrotron Radiation Facility) in Grenoble, were processed using DENZO and SCALEPACK [26]. Initial data on the dGMP complex were collected on the ID24 microfocus beam line at the Diamond Light Source (Didcot, U.K.) from a crystal conglomerate using an 8 × 8 μm2 beam to hunt for a single lattice with the help of Gwynyad Evans, Robin Owen and Danny Axford. This led to a 70% complete data set at 2.7 Å resolution and allowed the presence of the ligand to be confirmed. Subsequent optimization led to larger crystals from which complete data were
were modelled into a non-averaged 2\(F_o - F_c\) electron density map. After several rounds of refinement and rebuilding, the dTMP and ADP molecules were built into the electron density in the averaged map. Towards the end of model building, the dTMP and ADP molecules were built into all four complexes, before describing the structures in detail in the subsequent sections.

The structure of PfTMPK is a homodimeric protein containing 210 amino acid residues per subunit. All four nucleotide complexes crystallized in space group \(P 3_1 2 1\) with three protomers per asymmetric unit, recorded at the ESRF, but the power of beam line I24 for analysing tiny crystals was clearly demonstrated by this result.

Structure solution and refinement

The structure of PfTMPK complexed with dTMP and ADP was solved by molecular replacement with the program PHASER [27] using hTMPK (human TMPK, PDB code 1E2Q) as a search model (the two proteins share 39% sequence identity). Searches, carried out in two possible space groups (\(P 3 1 2 1\) and \(P 3 2 1\)), yielded solutions in space group \(P 3 1 2 1\) only and established the presence of three molecules per asymmetric unit. Refinement was carried out in the CCP4 suite of programs [28] using maximum likelihood methods implemented in REFMAC [29]. 5% of the data were excluded for \(R\)-free calculations. The models were manually rebuilt using the program COOT [30] in conjunction with a 3-fold averaged \(2F_o - F_c\) electron density map and a \(F_c\) electron density map. After several rounds of refinement and model building, the dTMP and ADP molecules were built into the electron density in the averaged map. Towards the end of the refinement, small differences between the three molecules were modelled into a non-averaged \(2F_o - F_c\) electron density map and a \(F_c\) electron density map. After several rounds of refinement and rebuilding, the dTMP and ADP molecules were built into all four complexes, before describing the structures in detail in the subsequent sections.

The three protomers are essentially identical to one another, with rmsds (root mean square positional deviations) following superposition of all \(C_v\) atoms of \(\sim 0.25\) Å (1 Å = 0.1 nm). Each subunit has a globular structure consisting of a five-stranded parallel \(\beta\)-sheet (\(\beta_2-\beta_3-\beta_1-\beta_4-\beta_5\)) surrounded by eight \(\alpha\)-helices (Figure 1b). Interaction with the partner subunit in the dimer occurs predominantly through hydrophobic interactions between \(\alpha\)-helices 2, 3 and 5 from each subunit (results not shown). The molecules are well-ordered, with only some small loop and chain termini disorder. Superposition of protomer A from all four complexes shows the backbone conformation to be very similar (Supplementary Figure S1 available at http://www.BiochemJ.org/bj/428/bj4280499add.htm) and corresponds to the closed form of the enzyme, as expected for complexes with both nucleotides.

RESULTS AND DISCUSSION

Kinetic parameters and inhibition of PfTMPK

Standard procedures were used to determine the kinetic parameters of recombinant PfTMPK. Plots of specific activity against substrate concentration gave typical Michaelis–Menten hyperbolic saturation curves. Kinetic constants were determined for a number of different substrates used as phosphoacceptors (Table 1). ATP was used as a phosphate donor in the assays, because it was the preferred donor among those tested (results not shown). The phosphorylating efficiency \(k_{\text{cat}}/K_m\) of PfTMPK was highest for dTMP followed by dGMP, 5FdUMP, dUMP and GMP. In addition, the \(K_m\) for ATP was 78.6 \(\mu\)M. This value was obtained at 200 \(\mu\)M dTMP and constant free Mg\(^{2+}\) concentrations (5 mM). These results are in general agreement with those reported previously [19], with the relative activities having the same rank order although the two assays differ in the absolute measured values. Although the assay methodology used in both cases is similar, subtle differences may be due to the different expression systems employed or minor differences in the reaction conditions. It is of particular note that the activity is almost as high for the purine dGMP as it is for dTMP. Surprisingly, phosphorylation of the nucleotide analogue AZT-MP was also highly efficient with a \(k_{\text{cat}}/K_m\) close to half that for dTMP. This latter value for AZT-MP is 200-fold higher than the corresponding value for hTMPK and similar to that of the E. coli enzyme (Table 2).

There was no measurable phosphorylation of the nucleosides uridine, thymidine, deoxyuridine and AZT by PfTMPK. Likewise, purine derivatives such as ganciclovir \([9\text{-}((1,3\text{-dihydroxy}-2\text{-propoxy})\text{ methyl}]\text{ guanine}]\) and 3′-deoxyguanosine-5′-monophosphate were not substrates. Different nucleotides and nucleosides were tested as inhibitors of dTMP phosphorylation. 5-fluoro-2′-deoxyuridine and 3′-deoxyguanosine-5′-monophosphate showed \(K_i\) values of 1.6 mM and 0.25 mM respectively, whereas no inhibition was observed with deoxyuridine, 5-fluoro-5′-deoxyuridine or ganciclovir. The \(K_i\) for AP5dT, a bi-substrate inhibitor of several TMPKs [13] was measured by determining the percentage of inhibition at different inhibitor concentrations and assuming competitive inhibition, giving a value of 0.31 \(\mu\)M. The key findings of the kinetic studies are the high activity of PfTMPK on AZT-MP and confirmation of its activity on dGMP. These activities will be discussed in terms of the structural investigations below.

Three-dimensional structure

PfTMPK is a homodimeric protein containing 210 amino acid residues per subunit. All four nucleotide complexes crystallized in space group \(P 3 1 2 1\) with three protomers per asymmetric unit, equating to one crystallographically independent dimer, plus a second dimer lying on a crystallographic two-fold axis. We first give a generic description of those features which are common to all four complexes, before describing the structures in detail in the subsequent sections.

The three protomers are essentially identical to one another, with rmsds (root mean square positional deviations) following superposition of all \(C_v\) atoms of \(\sim 0.25\) Å (1 Å = 0.1 nm). Each subunit has a globular structure consisting of a five-stranded parallel \(\beta\)-sheet (\(\beta_2-\beta_3-\beta_1-\beta_4-\beta_5\)) surrounded by eight \(\alpha\)-helices (Figure 1b). Interaction with the partner subunit in the dimer occurs predominantly through hydrophobic interactions between \(\alpha\)-helices 2, 3 and 5 from each subunit (results not shown). The molecules are well-ordered, with only some small loop and chain termini disorder. Superposition of protomer A from all four complexes shows the backbone conformation to be very similar (Supplementary Figure S1 available at http://www.BiochemJ.org/bj/428/bj4280499add.htm) and corresponds to the closed form of the enzyme, as expected for complexes with both nucleotides.

In the dTMP-ADP, AP5dT and dGMP complexes, there are two peaks at essentially identical positions in the electron density maps adjacent to the terminal phosphate groups of the nucleotides, whose shape and co-ordination indicated the presence of two metal ions. These were initially introduced as magnesium ions whose shape and co-ordination indicated the presence of two metal ions. These were initially introduced as magnesium ions and independent co-ordination spheres. In retrospect, this is not surprising as although 25 mM magnesium chloride was present.
Table 2  Kinetic parameters of TMPKs from different organisms for dTMP and AZT-MP

<table>
<thead>
<tr>
<th>Organism</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ · mM$^{-1}$)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_m$ (μM)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ · mM$^{-1}$)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>15</td>
<td>2.7</td>
<td>5600</td>
<td>6</td>
<td>30</td>
<td>200</td>
<td>28</td>
</tr>
<tr>
<td>S. cerevisiae</td>
<td>35</td>
<td>9</td>
<td>3900</td>
<td>0.175</td>
<td>6</td>
<td>29</td>
<td>130</td>
</tr>
<tr>
<td>Plasmodium falciparum</td>
<td>4.9</td>
<td>10.7</td>
<td>460</td>
<td>1.8</td>
<td>9.1</td>
<td>200</td>
<td>2.3</td>
</tr>
<tr>
<td>Human, wild-type TMPK</td>
<td>0.73</td>
<td>6.3</td>
<td>120</td>
<td>0.012</td>
<td>12</td>
<td>1.0</td>
<td>120</td>
</tr>
<tr>
<td>Human, TMPK-F105Y</td>
<td>0.17</td>
<td>4.2</td>
<td>40</td>
<td>0.25</td>
<td>3.8</td>
<td>66</td>
<td>0.61</td>
</tr>
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Table 3  Data processing and refinement statistics for the PfTMPK complexes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>dTMP–ADP</th>
<th>AP$_d$ dT</th>
<th>AZT-MP–ADP</th>
<th>dGMP–ADP</th>
</tr>
</thead>
<tbody>
<tr>
<td>ESRF beamline/wavelength (Å)</td>
<td>ID23-1 / 0.9184</td>
<td>ID14-4 / 0.9765</td>
<td>ID14-4 / 0.9395</td>
<td>ID23-1 / 0.9760</td>
</tr>
<tr>
<td>Data collection statistics</td>
<td>resolution limits (Å)</td>
<td>a = 110.13 c = 120.10</td>
<td>a = 109.54 c = 119.35</td>
<td>a = 110.34 c = 119.11</td>
</tr>
<tr>
<td>P 3; 2 1 cell dimensions (Å)</td>
<td>40.0 – 1.96 (1.93 – 1.90)</td>
<td>40.0 – 2.70 (2.80 – 2.70)</td>
<td>40.0 – 3.00 (3.11 – 3.00)</td>
<td>50.0 – 2.4 (2.49 – 2.40)</td>
</tr>
<tr>
<td>Number of unique reflections</td>
<td>67,190</td>
<td>23,265</td>
<td>17,385</td>
<td>33,557</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>94.3 (50.6)</td>
<td>99.2 (90.2)</td>
<td>99.7 (99.1)</td>
<td>99.4 (98.6)</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>51 (2.0)</td>
<td>8.1 (3.7)</td>
<td>9.4 (5.6)</td>
<td>10.9 (10.4)</td>
</tr>
<tr>
<td>R-merge</td>
<td>0.083 (0.298)</td>
<td>0.121 (0.481)</td>
<td>0.115 (0.287)</td>
<td>0.150 (0.944)</td>
</tr>
<tr>
<td>Refinement statistics</td>
<td>R-cryst/R-free (%)</td>
<td>16.04 / 20.34</td>
<td>18.67 / 27.00</td>
<td>19.93 / 28.69</td>
</tr>
<tr>
<td>rmsd bond lengths (Å)</td>
<td>0.025 (0.022)</td>
<td>0.014 (0.022)</td>
<td>0.013 (0.022)</td>
<td>0.020 (0.022)</td>
</tr>
<tr>
<td>rmsd angles (°)</td>
<td>2.007 (1.981)</td>
<td>1.655 (1.977)</td>
<td>1.585 (1.972)</td>
<td>1.860 (1.922)</td>
</tr>
<tr>
<td>rmsd chiral volume (Å$^3$)</td>
<td>0.224 (0.200)</td>
<td>0.102 (0.200)</td>
<td>0.107 (0.200)</td>
<td>0.131 (0.200)</td>
</tr>
<tr>
<td>Ramachandran outliers (%)</td>
<td>1.3</td>
<td>1.6</td>
<td>2.9</td>
<td>1.9</td>
</tr>
<tr>
<td>$B_{averg}$ on all atoms (Å$^2$)</td>
<td>20.0</td>
<td>37.5</td>
<td>32.1</td>
<td>43.4</td>
</tr>
</tbody>
</table>

in the crystalization drops, sodium is also present at large excess, ~1–2 M. These sodium ions in PfTMPK, primarily due to their larger ionic radius, occupy somewhat different positions to those observed for the “biological” magnesium ions seen in the human enzyme (Figure 2c), and some other known structures, which correspond to the two metal ions essential for phospho group transfer. Equivalent sodium ions are likely to be present, but were not modelled, in the lower resolution AZT-MP complex. The sodium ions have rather different co-ordination from the magnesium ions, as described in the next section, with bonds formed to the nucleotides and protein residues.

PfTMPK has the same general fold and mode of substrate binding as other TMPKs with known structures (see http://www.ebi.ac.uk), suggesting a common mode of action. Thus PfTMPK would be expected to bind the two nucleotide co-substrates independently of one another, with binding accompanied by stepwise closure of the P-loop and lid so that phosphate transfer can be facilitated by magnesium ions, arginine residue side chains and other essential conserved residues.

A superposition of the dTMP–ADP complex of PfTMPK on the equivalent complex of the human enzyme shows that the cores of the molecules are very similar (see below), with some minor differences evident in the loops remote from the active site, and some significant deviations in the loops close to the active site (Supplementary Figure S2 available at http://www.BiochemJ.org/bj/428/bj4280499add.htm). A set of substates of the closed form of the human enzyme, with the lid and P-loop being either in the open, half-closed or closed form, depending on the ligand bound, have been described [15].

Comparison of the backbone conformation of PfTMPK with that of the human enzyme complexes shows that it corresponds most closely to the closed sub-conformation of the lid and P-loop (Supplementary Figure S3 available at http://www.BiochemJ.org/bj/428/bj4280499add.htm). Indeed in terms of the ‘degree of closure’, PfTMPK appears to be even more tightly closed around the nucleotides in the four complexes than in the equivalent human structures. In summary, the comparison of PfTMPK and hTMPK reveals significant and consistent conformational changes in the catalytically important P-loop and lid regions. These conformational differences, together with natural side-chain variations, may account for differences in specificity and rate in the two enzymes.

PfTMPK–dTMP–ADP complex

The structure of the PfTMPK–dTMP–ADP complex was refined at a resolution of 1.9 Å. A prominent groove on the surface of each subunit forms the binding site for dTMP and the preferred phosphate donor, ATP, which bind co-linearly to facilitate phosphate group transfer. Residues of the lid region flank the bound nucleotides, partially enclosing the binding site. The presence of both dTMP (substrate) and ADP (product) results, as stated above, in the closed form of the enzyme with the lid fully ordered. The dTMP is buried, the thymine base being maintained in position by hydrogen bonds (both directly and indirectly via ordered water molecules) to the side chains of Arg$^78$ and Tyr$^{153}$, and a ring stacking interaction with Phe$^{24}$.  

$\text{Ratio} = \frac{k_{cat}/K_m}{d\text{TMP}/AZT-MP}$
Figure 2  Structure of the PfTMPK dTMP-binding site, PfTMPK ADP-binding site and position of metal ions

(a) Stereo images of the dTMP-binding site in the PfTMPK dTMP–ADP complex (protein and solvent coloured blue, dTMP by atom type) superimposed on the equivalent human complex (PDB code 1E2D, yellow). (b) Stereo image of the ADP-binding site. The position of the ADP base is displaced somewhat, due to shifts in the lid and P-loops, the more tightly closed PfTMPK structure perhaps reflecting the binding of sodium ions. (c) Stereo image of superposition of magnesium and sodium ions in the human (yellow ribbon) and PfTMPK complexes (blue) respectively, with associated 2Fo–Fc electron density contoured at 1σ. Sodium ions are shown as grey spheres, magnesium ions in black and water molecules in red.

(Figure 2a). The ribose ring is loosely packed between the side chains of Tyr153 and Arg99, and forms one direct hydrogen bond to Asp17 in the P-loop. There is clear evidence for the binding of two sodium ions (Figure 2c). The phosphate group makes direct interactions with the side chain of Arg99 and sodium ion (I), which is penta-co-ordinate, its ligands consisting...
of a phosphate oxygen (from dTMP), the side-chain oxygen atoms of Asp$^{17}$ and Glu$^{14}$ and two water molecules (Figure 2c). The existence of a third highly mobile water molecule to complete the octahedral co-ordination sphere cannot be excluded. Arg$^{99}$, which forms part of the highly conserved critical loop, is the only residue in the structure whose main-chain bond angles lie in a disallowed region of the Ramachandran plot. This phenomenon has been observed in other TMPKs, and can be presumed to be associated with catalysis as steric strain in well-refined crystal structures correlates with functional significance [32]. The residues involved in dTMP binding are highly conserved and superposition of the dTMP-bound TMPK structures available from the PDB shows that the mode of binding is very similar in each.

The ADP, bound close to the surface of the protein, is protected by the lid and is secured in position by a number of interactions (Figure 2b). These include stacking between the adenine base and the guanidine moiety of Arg$^{148}$, a hydrogen bond between the base and the main-chain oxygen of Arg$^{142}$, three hydrogen bonds between the phosphate groups and the atoms of residues Lys$^{21}$, Ser$^{15}$ and Thr$^{15}$ (P-loop), and a direct interaction with sodium ion (II). Sodium ion (II) has an octahedral co-ordination sphere comprising one phosphate oxygen from ADP, the side-chain carboxylate of Asp$^{98}$, the side-chain oxygen atom of Ser$^{108}$ and three water molecules. The catalytically important Asp$^{15}$ of the P-loop has a full complement of polar interactions, including hydrogen bonds to Tyr$^{107}$ and Gln$^{109}$. The side chain of the adjacent Arg$^{18}$ points away from the substrate, hydrogen bonding to the side chain of Glu$^{148}$.

Superposition of the structure of P/P TMPK–dTMP–ADP with the equivalent complex of hTMPK (PDB code 1E2D; sequence identity 40%; sequence similarity 60%) shows a highly conserved topology (rmsd of 1.35 Å over 195 C$\alpha$ atoms) and mode of nucleotide binding (Supplementary Figure S2); the dTMP-binding sites are essentially identical. However, there are small but significant differences in the lid regions, which differ in conformation, and in the P-loops, which have a relative displacement of 2.1–2.5 Å on main chain atoms, and also differences in side-chain conformations. For the human enzyme, the P-loop displacement results in a shift in position of the phosphate groups of ADP and a different mode of metal binding reflecting the presence of sodium ions rather than magnesium ions (Figure 2c). The different conformation for Asp$^{15}$ results in magnesium ion (I) in hTMPK having a quite different co-ordination sphere, consisting of six water molecules and an indirect rather than direct interaction with dTMP, compared with that of sodium ion (I) in P/P TMPK (Figure 2c). A probable reason for the difference in the position of the P-loop is a natural variation in the substrate-binding site, Phe$^{100}$ (human)→Tyr$^{107}$ (Plasmodium). The tyrosine side chain of P/P TMPK forms a hydrogen bond (2.6 Å) with the side chain of Asp$^{17}$, which helps to tether this catalytically important residue in close proximity to the terminal phosphate groups of the nucleotide co-substrates (Supplementary Figure S4 available at http://www.BiochemJ.org/bj/428/bj4280499add.htm). The phenylalanine residue in the human enzyme lacks the capacity to do this. There is a tyrosine at this position in yeast TMPK and this residue has been cited as the reason for its enhanced catalytic activity relative to hTMPK [33]. The other P-loop side chain believed to be important for catalysis, Arg$^{18}$ (Arg$^{16}$ in hTMPK), is directed away from the nucleotide-binding site in P/P TMPK and hTMPK, in both structures making hydrogen bonding interactions with lid residues. Residue Lys$^{37}$ in yeast TPK corresponds to Arg$^{41}$ and Tyr$^{41}$ in the human and P. falciparum enzymes respectively (Figure 1a). These natural variations in the substrate-binding site may provide scope for the design of selective inhibitors against P/P TMPK.

### Structural basis for dGMP phosphorylation

In view of the kinetic observation that P/P TMPK phosphorylates dGMP with a k$_{cat}$/K$_m$ value close to that for dTMP, we determined the structure of the dGMP–ADP complex, which was refined to a resolution of 2.4 Å. Two sodium ions were modelled at equivalent positions to those in the dTMP complex, but with reduced electron densities reflecting the lower resolution of the analysis. The guanine nucleotide is accommodated in the pyrimidine-binding pocket (Figure 3a) with negligible changes in the protein structure compared with the dTMP–ADP structure (rmsd of 0.25 Å over 209 C$\alpha$ atoms). The guanine base is co-planar with the dTMP thymine and just requires the displacement of a single highly ordered water molecule, which in the dTMP–ADP complex hydrogen bonds to the second oxygen atom of thymine and the hydroxy groups of residues Tyr$^{153}$ and Ser$^{108}$. The ribose and the phosphate groups of dGMP have closely similar conformations and positions to those of dTMP and the ADP-binding sites are essentially identical.

The structure of the human dTMP–ADP complex is superimposed on the P/P TMPK complex in Figure 3(a). From the close similarity of the two structures, it might be expected that the human enzyme would also bind dGMP. However, in spite of the fact that the dTMP-binding site of hTMPK is very similar to that of P/P TMPK, particularly around the pyrimidine-binding pocket, several studies have demonstrated that dGMP is not a substrate for the human enzyme [34–36]. In human cells it appears that a guanylate kinase very efficiently phosphorylates dGMP and GMP [36]. A survey of the P. falciparum genome (see http://www.PlasmoDB.org) shows the existence of a guanylate kinase and a UMP–CMP kinase which have potential functions in dGMP phosphorylation, as guanylate kinases are capable of dGMP phosphorylation [37], likewise certain UMP kinases, such as that from yeast, have been shown to phosphorylate dGMP [38]. A recent study has revealed that the P. falciparum guanylate kinase utilizes dGMP with very low specificity, which is of interest in respect to the potential contribution of P/P TMPK to cellular GDP formation and purine nucleotide metabolism in the parasite [39].

Site-directed mutagenesis has been used previously to evaluate the contributions of selected residues to substrate-binding and catalysis [20]. The mutation of the conserved Phe$^{42}$, whose side chain stacks with the thymine base, to an alanine residue eliminated activity almost entirely. Introduction of individual point mutations between the human and parasite enzymes (Figure 1a) into P/P TMPK (S108T, Y43R and Y43L) had negligible effects on activity. The structure of P/P TMPK reveals that although these residues are close to the active site, they are not directly involved in nucleotide binding. Replacement of Ala$^{111}$ by a lysine residue, resulted in a dramatic reduction in the activity of P/P TMPK, especially in dGMP phosphorylation. Ala$^{111}$ resides on a seven-residue loop on the outside of the molecule, which has quite different conformations in the two enzymes. As a result, Ala$^{111}$ and Lys$^{109}$, rather than coinciding, are displaced by 3.7 Å from one another (C$\alpha$), and located 10–12 Å away from the dTMP ligand exerting no direct influence on binding. Indeed the A111K mutation in P/P TMPK would be expected to cause a steric clash with the side chain of Tyr$^{153}$, which is in van der Waals’ contact with both the base and the sugar of dTMP. Displacement of the tyrosine side chain would be predicted to affect substrate binding, and this would be exacerbated for the larger dGMP, in keeping with the kinetic results.

Vaccinia virus TMPK is also capable of phosphorylating purine nucleotides [34] and recent structural analysis has revealed a
dimer arrangement that is orthogonal, and not anti-parallel, as in other TMPKs. This unusual subunit tilting is closely correlated with enzyme stability and with the presence of a cavity allowing broader substrate specificity [40]. Apart from 

*Plasmodium* and vaccinia TMPKs, the only other kinase that is known to handle both dGMP and dTMP is the bacteriophage T4 deoxynucleotide kinase. The structure of the phage enzyme is similar to the fold of the NMP kinases, but with a different dimerization mode and no apparent conservation of the active-site residues [41].

**PfTMPK–AP5dT complex**

The bisubstrate inhibitor AP$_5$dT [13] has been co-crystallized with TMPK from a number of species [6,14,15]. In the PfTMPK complex, AP$_5$dT is bound at full occupancy with the same mode of binding in all three protomers (Figure 3b). The five phosphate groups of the inhibitor are labelled PA–PE, phosphate PA being closest to the thymidine group. Superposition with the PfTMPK–TMP–ADP complex shows that the effect of bisubstrate binding on the protein conformation is minimal, with an rmsd for all main-chain atoms of 0.3 Å. The adenosine and thymidine moieties of AP$_5$dT superpose closely with the nucleoside species of ADP and dTMP, as do the phosphates PA, PD and PE with the phosphate of dTMP and the β- and α-phosphates of ADP respectively. The two bridging phosphate groups of AP$_5$dT, PB and PC, are bound next to the P-loop, flanked by the two sodium ions. The non-bridging oxygens of all five phosphate groups make polar interactions with Lys$^{23}$, Ser$^{22}$ (main chain and side chain respectively), Thr$^{23}$ (main chain), Arg$^{47}$ and Arg$^{99}$ or with the metal ions. In addition, the side chain of Arg$^{18}$ has two alternate conformations, one of which...
hydrogen bonds to a phosphate oxygen of PC. The presence of this interaction is consistent with a role for Arg18 in transition-state stabilization, as has been shown for the yeast and human enzymes. Two sodium ions were modelled in the structure, and lie approx. 0.7 Å from the equivalent ions in the dTMP–ADP complex.

Superposition of PfTMPK–AP$_5$dT with the equivalent complexes from other species (Figure 3b) confirms that the inhibitors bind in the same place, although only the thymidine moieties are truly co-incident. The atoms of the five phosphate groups and the adenosine moieties are displaced by 0.3–1.2 Å (one of the phosphates is ordered in the human complex). The sodium ion (I) and magnesium ion (I) lie 2.1 Å apart in the human and Plasmodium enzymes, although they carry out the same role, co-ordinating to two oxygen atoms of phosphates PC and PD. The P-loops of the two structures overlay more closely than they do in the dTMP–ADP complex, although there are still main-chain atom differences of 0.8–1.7 Å. The side chains of Asp$^{17}$ (Asp$^{15}$ in hTMPK) overlap but the carboxylates make different hydrogen-bonding interactions, to Tyr$^{107}$ in PfTMPK and to Arg$^{97}$
in hTMPK. The P-loop arginine side chains are arranged much as they are in the dTMP–ADP complex, with the exception of the dual conformation of the side chain of Arg18 in PfTMPK. The binding of AP_dT clearly varies in detail between species, but is in keeping with the accepted mechanism of the enzyme.

**PfTMPK efficiently phosphorylates AZT-MP**

Another characteristic of PfTMPK highlighted by the present kinetic studies is its ability to phosphorylate AZT-MP 200-fold more efficiently than hTMPK (Table 2). The conversion of AZT-MP into AZT-DP (AZT diphosphate) by the human enzyme is a crucial step in the production of the active drug AZT-TP (AZT triphosphate) in antiviral therapy. The antiviral effect of the drug is achieved by its inhibition of nascent DNA chain growth during replication following its acceptance as a substrate by the viral DNA polymerase [8].

Analysis of the PfTMPK–AZT-MP–ADP complex shows that both nucleotide substrates are at full occupancy in each protomer in the asymmetric unit, however only in molecule A can the position of the azide group be defined with confidence (Figure 3c). The absence of electron density for azide in the other two protomers may reflect inherent disorder and is exacerbated by the modest (3.0 Å) resolution. The AZT-MP azide moiety in molecule A is accommodated in a cavity surrounded by the side chains of Asp17, Leu59, Glu151 and Glu154 (Figure 3d). It causes a small reorientation of the dTMP phosphate group and minor conformational changes in the side chains of Glu151 and Asp17, the latter retaining its hydrogen bond to the side chain of Tyr107. Owing to the limited resolution, metal ions were not modelled in this complex (Table 3). The structure superimposes very closely on the dTMP–ADP complex (rmsd of 0.27 Å over 208 Cα atoms) and the positions of the two sets of nucleotide ligands are essentially identical.

Comparison with the structure of the equivalent human enzyme complex (PDB entry 1E99) shows differences in the P-loop organization similar to those observed in the equivalent dTMP–ADP comparisons. The close proximity of the side chain of Arg18 to the AZT-MP in PfTMPK causes a reorientation of the azide group relative to that in the human complex. This reorientation does not appear to affect the availability of Asp17 for catalysis. Indeed, given that it is likely that no further P-loop changes are required for transition-state formation in the P. falciparum enzyme (compared with the structure of PfTMPK–AP_dT) it would appear to have a mechanistic advantage over the human enzyme, in which P-loop movements toward the transition state would result in a steric clash with the azide group of AZT-MP.

These structural observations provide a likely explanation for the higher activity of PfTMPK relative to hTMPK towards AZT-MP (Table 2).

Poor AZT-MP phosphorylation in the type I TMPKs, such as the yeast and human enzymes, which contain a basic residue in the P-loop has been proposed to be due to the azido moieties in AZT-MP disrupting an important bidentate interaction between the 3′ hydroxy group of TMP and the conserved P-loop carboxylic acid which results in a displacement of the P-loop [8]. This is in contrast with type II TMPKs, such as the E. coli enzyme, where the analogous interaction with the P-loop carboxylic acid residue is side-on, permitting small conformational changes to accommodate the azido group of AZT-MP without affecting significantly the position of the P-loop. It has been proposed that the P-loop could influence the phospho transfer rate of type I TMPKs in different ways [11]. First, by shifting the position of ATP relative to the acceptor, catalysis would be impaired. Secondly, movement of the P-loop upon binding of AZT-MP would result in the mis-positioning of P-loop arginine involved in catalysis (Arg18 in PfTMPK). Thirdly, mis-positioning of the P-loop carboxylic acid residue, a strictly conserved amino acid within the TMPK family, may hinder catalysis [18]. The steric clash between the azide group and the P-loop as the latter closes over the substrate is not likely to occur in PfTMPK as hydrogen bonding between Asp17 and Tyr107 places the P-loop in the fully closed position at an earlier stage in the reaction, causing the azide group to adopt a different orientation (Figure 3c), which should not interfere with catalysis. This significance of this tyrosine residue for AZT-MP activation is supported by kinetic results on the hTMPK-F105Y mutant [18,33] which is much more tolerant of AZT-MP than the wild-type enzyme (Table 2).

In summary, despite the overall conservation of sequence and structure in the nucleotide-binding site of human and P. falciparum TMPK, it is apparent that there are a number of subtle but seemingly significant differences in the substrate and inhibitor specificities. These are highlighted by differences in the reaction catalysed by the two enzymes with dGMP and AZT-MP, evidence to suggest that PfTMPK may indeed be exploited as a target for anti-malarial drug discovery.

**AUTHOR CONTRIBUTION**

The project management team responsible for experimental design and co-ordination of research activities comprised Dolores González-Pacanowska, Keith Wilson, Ian Gilbert and Anthony Wilkinson. Jean Whittingham determined the X-ray structure of PfTMPK and conducted the structural studies, with contributions from James Brannigan and Ana Silva, who solved the dGMP structure. Luís Ruiz-Pérez and Juana Carrero-Lerida were responsible for parasite culture, kinetic analyses and inhibitor studies. Mark Fogg, Luís Ruiz-Pérez and Juana Carrero-Lerida were involved in gene cloning, protein expression and purification. All authors analysed results and contributed to writing the paper.

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SUPPLEMENTARY ONLINE DATA

Structural basis for the efficient phosphorylation of AZT-MP (3′-azido-3′-deoxythymidine monophosphate) and dGMP by Plasmodium falciparum type I thymidylate kinase

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Figure S1 Structure of the P-loop and lid for all four PfTMPK–ligand complexes

Superposition of the region including P-loop and lid of all four PfTMPK ligand complexes reported in the present paper (dTMP–ADP, grey; AP5dT, red; AZT-MP, blue; dGMP–ADP, green): the backbone conformation is very similar in all four. The nucleotides, metals and associated waters are shown for the dTMP–ADP complex.

Figure S2 Structure of the PfTMPK–TMP–ADP complex

Stereo image of the PfTMPK–TMP–ADP protomer (blue coil) superimposed upon that of hTMPK–TMP–ADP (coral, PDB code 1E2Q). The associated dTMP and ADP ligands are shown in ball and stick for PfTMPK coloured by atom type, and for hTMPK in coral. The metals ions are shown as spheres, with the PfTMPK sodium ions in grey and human magnesiums ion in coral.

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The structures for the four PfTMPK–ligand complexes will appear in the PDB under codes 2WWF (with dTMP–ADP), 2WWG (with dGMP–ADP), 2WWH (with AP5dT) and 2WWI (with AZT-MP–ADP).
Figure S3  Structure of the PfTMPK–TMP–ADP complex compared with hTMPK–ligand complexes

Stereo image of the superposition of the PfTMPK–TMP–ADP protomer (black) with the structures of five complexes of human TMPK: dTMP–ADP (PDB code 1E2D, red), TP5A (PDB code 1E2Q, green), dTMP–ADP (PDB code 1E2F, blue), dTMP–ADP–AlF₃ (PDB code 1E2E, coral) and dTDP–ADP (PDB code 1E2G, magenta). The residues Glu₁₅⁴ and Asp₁⁷ are shown as stick representations and coloured by atom.

Figure S4  Structure of the PfTMPK dTMP–ADP-binding site

Detailed image view of the PfTMPK dTMP–ADP site: selected residues of the P-loop are shown to highlight the differences in these parts of the structures (PfTMPK, blue; hTMPK, yellow). A hydrogen bonding interaction between the side chains of Asp₁⁷ and Tyr₁₀⁷ in the PfTMPK enzyme is shown.
Table S1  Crystallization conditions for the four *PfTMPK* nucleotide complexes

Conditions are adapted from Index Screen, Hampton Research.

<table>
<thead>
<tr>
<th>Complex</th>
<th>dTMP–ADP</th>
<th>AP5dT</th>
<th>AZT-MP–ADP</th>
<th>dGMP–ADP</th>
</tr>
</thead>
<tbody>
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<td>Drop</td>
<td>10 mg·ml⁻¹ protein in 50 mM Tris/HCl, pH 8.5, containing 50 mM NaCl, 2 mM dTMP, 2 mM ADP and 50 mM MgCl₂.</td>
<td>10 mg·ml⁻¹ protein in 50 mM Tris/HCl, pH 8.5, containing 50 mM NaCl, 2 mM AP5dT and 50 mM MgCl₂.</td>
<td>10 mg·ml⁻¹ protein in 50 mM Tris/HCl, pH 8.5, containing 50 mM NaCl, 2 mM ADP, 2 mM AZT-MP and 50 mM MgCl₂.</td>
<td>10 mg·ml⁻¹ protein in 50 mM Tris/HCl, pH 8.5, containing 200 mM NaCl, 2 mM dGMP, 2 mM ADP and 50 mM MgCl₂.</td>
</tr>
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<td>Reservoir</td>
<td>75 mM Hepes, pH 7.5, containing 1.35 M tri-sodium citrate and 0.125 M NaCl.</td>
<td>2.04 M sodium malonate, pH 7.0</td>
<td>75 mM Mes, pH 6.5, containing 1.35 M tri-sodium citrate and 0.125 M NaCl.</td>
<td>4 mM Hepes, pH 7.5, containing 1.32 M tri-sodium citrate, 80 mM NaCl and 3% (w/v) xylitol</td>
</tr>
<tr>
<td>Cryo-protectant</td>
<td>40% (v/v) glycerol and 60% (v/v) reservoir solution</td>
<td>3.0 M sodium malonate</td>
<td>40% (v/v) glycerol and 60% (v/v) reservoir solution</td>
<td>50 mM Tris/HCl, pH 8.5, containing 200 mM NaCl, 1 mM ADP, 1 mM dGMP, 50 mM MgCl₂, 3% (w/v) xylitol, 25% (v/v) glycerol and 1 M tri-sodium citrate</td>
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